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Evaluation of kinetics and single-read enzyme-linked immunoassays for detection of *Toxoplasma gondii* antibodies in sheep

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Abstract. A kinetics enzyme-linked immunosorbent assay (ELISA) and a single-read ELISA for the detection of ovine anti-*Toxoplasma gondii* IgG were developed and optimized. During the kinetics assay, 3 optical densities were obtained for each serum sample at intervals of 45 seconds, and the results were presented as average slopes (replicates of 2) of the reaction rate between bound enzyme and substrate solution. The kinetics ELISA was stopped 5 minutes after dispensing the substrate to constitute the single-read ELISA, and the results were presented as average optical densities for duplicates of each sample. Performance of the assays was evaluated using the modified agglutination test (MAT) as the ''gold standard.'' There was a high level of agreement between both ELISAs and the MAT, as measured by Pearson correlation coefficients, kappa statistics, and the area under the receiver operating characteristics curves. The single-read ELISA was as accurate as the kinetics ELISA, with a sensitivity of 89% and a specificity of 96%.

Ovine toxoplasmosis remains an important cause of abortion and neonatal mortality in sheep in many countries.[1] In the United States, prevalence rates >50% have been reported.[2,12] Routine diagnosis and epidemiologic studies of veterinary and medical diseases require the use of diagnostic tests or kits that have been validated for the target population.[9] Several diagnostic tests and kits have been developed for ovine toxoplasmosis, including the direct agglutination test,[18] dye test,[8,15] enzyme-linked immunosorbent assay (ELISA),[18,19,22] indirect hemagglutination test,[4] indirect immunoflourescent antibody test,[19] latex agglutination test,[25] modified agglutination test (MAT),[4] polymerase chain reaction,[16,27] and Western blotting.[6] A diagnosis of ovine toxoplasmosis can also be made through identification of pathologic changes and isolation of the parasite in mice.[1]

The kinetics ELISA (KELA) was based on a previously developed system.[10] In an ELISA, the indicator system for detection of antibody is conversion of a chromogen to a colored product in the presence of a specific bound enzyme and substrate. The KELA system measures the rate of reaction that leads to development of color. Variability in results is thus reduced because the readings are independent of the time of substrate addition. The slope of the reaction rate is also directly proportional to the amount of antibody present in the reaction vessel. There is a general perception among diagnosticians that the KELA is superior to the single-read ELISA.

The objectives of this study were to develop a KELA as a diagnostic test for exposure of sheep to *Toxoplasma gondii* and to compare the performance of the KELA to that of a single-read ELISA.

Materials and methods

ELISA development and standardization. The principle of the 2 tests, KELA and single-read ELISA, is the same. The indirect ELISA involves adsorbing the antigen onto the plate, washing, adding serum (test serum) containing antibodies to the antigen, washing, adding the enzyme-labeled anti-species (from which the test serum was derived) antibody, washing, and finally addition of substrate solution consisting of chromogen and substrate.

Toxoplasma gondii antigen[a] from tachyzoites of the RH strain was diluted 1:10 in sterile phosphate-buffered saline (PBS) followed by French pressing to release more antigen into solution. The protein concentration was determined to be 1,800 μg/ml, using a commercial protein assay kit[b] in a microtiter assay format according to the manufacturer's instructions. After processing, the antigen was aliquoted and stored at −70 C until used.

The conjugate used in the study was protein G[c] conjugated to horseradish peroxidase. After procurement, the conjugate was diluted 1:10 in sterile PBS, aliquoted, and stored at −20 C until used. A checkerboard titration was carried out to determine the optimum antigen concentration and conjugate dilution for use in the KELA and single-read ELISA. Known positive and known negative samples[d] were available for use in the checkerboard titration. Antigen concentrations considered in the study included 20, 10, 5, and 2.5 μg of antigen

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per milliliter of carbonate buffer (pH 9.6). The conjugate dilutions evaluated were 1:2,000, 1:4,000, and 1:6,000. All the dilutions were made with PBS containing 0.05% Tween 20 (PBS-T). The ability of milk to reduce background activity (blocking agent) was also investigated by diluting the conjugate in PBS-T containing 1% powdered milk. Maximal discrimination between the ELISA readings for the known positive and known negative serum samples, although retaining low background activity for the negative sample, was the criterion for selecting optimum reagent concentrations/dilutions. Samples dilutions were fixed at 1:100 in PBS-T based on experience.

Optimized protocol. Toxoplasma gondii antigen (5 μg/ml) in carbonate buffer (pH 9.6) was added to wells (200 μl/well) of high-binding microtiter plates.[e] The plates were then incubated at 4 C for at least 12 hr before use. Immediately before use, the plates were washed 4 times (30 sec for each wash) in PBS-T. Samples and standards were diluted 1:100 in PBS-T and dispensed into wells in duplicate. After incubation for 1 hr at 37 C, the wells were washed as described above. A solution of protein G conjugated to horseradish peroxidase[c] (diluted 1:6,000 in PBS-T) was dispensed at 100 μl/well and incubated for 30 min at 37 C. The plates were again washed as described above. Following the last wash, the PBS-T solution was left in the wells. Just prior to reading of the plates, the wash solution was aspirated, and 100 μl of 3,3′,5,5′-tetramethylbenzidine chromogen in 0.002 M H_2O_2 substrate was dispensed into each well. For the KELA, 3 optical density (OD) readings were taken at 45-sec intervals using the KELA data management program.[10] The results consisted of slopes representing the rate of change of color of the reaction between the conjugate enzyme, the substrate (H_2O_2), and the chromogen. The protocol for the single-read ELISA includes stopping the reaction (with 0.025 M hydrochloric acid) 5 min after dispensing the substrate, and the OD is read once. The KELA results were recorded at a wavelength of 680 nm, and that for the single-read ELISA was recorded at 450 nm. Standards and samples were processed in duplicate. As a quality control, a coefficient of variation (CV) of >10% among the standards would render the run (a run is equivalent to a plate) invalid. A CV of >10% among the samples would render the results for that particular sample during that run invalid.

Standards. Six serum standards were selected, aliquoted, and stored at −20 C for use in each run of the assay. The ELISA activity for all test samples was adjusted (normalized) by use of the standard curve so that all results were comparable between days and across plates within days. The standards prepared included a low negative, negative, low positive, medium positive, medium-high positive, and a high positive.

ELISA validation. To validate both the KELA and the single-read ELISA, a panel of reference serum samples (225 samples) was assembled from 5 farms in New York State. These samples were collected to determine the background (baseline) prevalence of ovine toxoplasmosis on the respective sheep farms before a 12-mo follow-up study of ovine toxoplasmosis. The samples were initially tested using an MAT,[3,4] and the relative amount of antibodies was determined. The samples were retested with the KELA and single-read ELISA, and the results are reported in terms of slope and OD, respectively.

Distribution of normalized slopes (from the KELA) and normalized ODs (from the single-read ELISA) were depicted as histograms and examined for peaks corresponding to seropositive and seronegative samples. A distribution-based cutoff point (to discriminate between positive and negative samples) for each of the assays was determined.

Indices of agreement of the tests. Correlations between slopes, ODs, and natural logarithm of MAT titers were assessed using Pearson correlation coefficients. The relationship between the results of the KELA and the MAT and the results of the single-read ELISA and the MAT was also examined using the linear regression analysis. Expected values (calculated as the mean from 40 runs in the follow-up study) for each of the 6 controls (low negative, negative, low positive, medium positive, medium-high positive, and high positive) were used in an inverse prediction to determine the corresponding log MAT titer. A 95% prediction interval was also determined. Relationships between the KELA and the single-read ELISA were noted. For both ELISAs, the distribution-based cutoff point was used to generate groups positive and negative for ovine toxoplasmosis. The MAT results were also categorized into positive and negative groups using an MAT titer of 25 as the cutoff point. A sample with an MAT titer of ≥25 was categorized as seroconversion to *T. gondii*, and samples with MAT titers of <25 were considered negative.[3,4] Agreements between the single-read ELISA and MAT results and between the KELA and MAT results were assessed using kappa statistics.

Indices of accuracy. The logistic regression approach was used to model the probability that a sample is positive for ovine toxoplasmosis (using an MAT titer of 25 as the cutoff) given a KELA slope or single-read OD. The model was specified as follows:

$$p(Y) = \frac{1}{1 + e^{\alpha+\beta x}}$$

where $p(Y)$ is the probability of seroconversion, Y is the event that the sample is positive for ovine toxoplasmosis, and x is the ELISA reading (slope for the KELA or OD for the single-read ELISA). To determine the trade-off between true-positive and false-positive rates of the test at each cutoff point, a receiver operating characteristic (ROC) curve was constructed by changing the cutoff point that determines which estimated event probabilities are considered to predict the event. For each cutoff point, the sensitivity and specificity were computed. The area under the ROC curve was calculated using the trapezoidal rule. This area is a measure of association of predicted probabilities and observed responses. For each of the assays, a cutoff point based on the estimated probability was determined from the respective ROC curve. From the estimated logistic regression equation, cutoff points based on slopes (for the KELA) and ODs (single-read ELISA) were determined. At each cutoff point, the sensitivity and specificity were noted as the indices of accuracy in addition to the ROC.

Serum samples from experimentally infected sheep. Sequentially collected serum samples[f] were available from 4 sheep. The sheep were inoculated orally with 1,000 (group

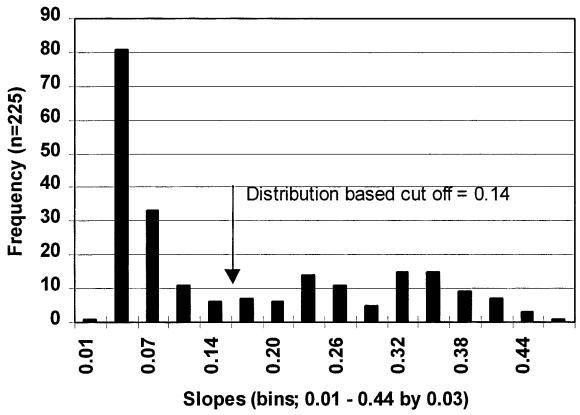

Figure 1. Frequency distribution of the KELA slopes derived from analysis of reference serum samples, showing the distribution-based optimal cutoff point.

1) or 10,000 (group 2) infective oocyts of the TS-2 isolate of *T. gondii*. Blood samples were collected from the jugular vein of each ewe 1–4 wk before inoculation, on the day of inoculation, and weekly thereafter until necropsy. Additional details concerning sheep inoculation and management have been described by Dubey et al.[4] These samples were used to examine the relationship between ELISA and time to seroconversion after infection.

Standard curves for the assays. The expected values (calculated as the mean from 40 runs in the follow-up study) for the 6 standards were incorporated into a standards file for each of the assays; the values were mean slopes of each standard for the KELA and mean ODs for the single-read ELISA. For each plate (run), the observed values for the 6 standards were regressed against the expected values in the control file (simple linear regression analysis). The regression equation can be expressed as follows:

$$y = \alpha + \beta x + e_{ij},$$

where y corresponds to the observed values for the 6 standards on a particular plate, x corresponds to the expected values for the 6 standards in the control file, and e corresponds to random error. All the observed sample ELISA (kinetics or single read) values for each plate were then converted into normalized values using inverse prediction. The resulting ELISA values could then be compared across runs and days quantitatively.

Levey-Jennings charts. In addition to the 6 standards maintained on each plate throughout the follow-up study, an independent standard was tested on each plate. After normalizing the results, ELISA values (kinetics or single read) were plotted against run to give Levey-Jennings charts.[9] These charts assess the repeatability of the assays. Differences and similarities were noted between the KELA and the single-read ELISA.

Results

ELISA optimization. The optimum antigen concentration was determined by checkerboard titration to be 5 μg/ml. Similarly, the appropriate conjugate dilution was determined to be 1:6,000 in PBS-T. Milk added to the conjugate diluent severely reduced the overall signal detected.

Table 1. Results of the analysis of KELA expected log MAT equivalent titers for the 6 standards.

Sample	Expected slope	Expected log MAT titer	95% prediction interval
Low negative	0.028	−1.16	−1.56, −0.78
Negative	0.052	−0.62	−1.02, −0.24
Low positive	0.112	0.75	0.10, 1.40
Medium positive	0.240	3.66	2.48, 4.87
Medium-high positive	0.370	6.63	4.98, 8.33
High positive	0.436	8.11	6.32, 9.97

ELISA validation. A total of 225 samples were tested by the MAT; 100 were positive. The results of the MAT were used as the ''gold standard.''

The frequency distribution of the KELA normalized slopes was trimodal (Fig. 1). The first peak, corresponding to the negative samples, was skewed to the right. The next 2 peaks, corresponding to the medium-positive samples (middle peak) and the high-positive samples (last peak), were approximately bell shaped. The distribution-based cutoff point for the KELA was chosen at the point were the slant on the right half of the first peak is first interrupted by a rise in frequency. This point corresponds to a KELA slope of 0.14.

The frequency distribution of the single-read ELISA normalized ODs was bimodal. The first peak, corresponding to the negative samples, was skewed to the right. The second peak, corresponding to all the positive samples, was approximately bell shaped, with a saddle at the top. The distribution-based cutoff point was determined to be 0.89 (using the same criterion as for KELA).

Indices of agreement. KELA slopes were highly correlated with log MAT titers; Pearson's correlation coefficient was 0.84. There was an equally high correlation between single-read ELISA ODs and log MAT titers (correlation coefficient = 0.84). The correlation between KELA and single-read ELISA results was also very high (correlation coefficient = 0.996). The linear relationships between the KELA slopes and log MAT titers and between the single-read ELISA ODs and log MAT titers were highly significant (P = 0.000).

The expected geometric mean MAT titers as predicted by the KELA were 0.31 for the low-negative standard and 3327 for the high-positive standard. The expected geometric mean MAT titers as predicted by the single-read ELISA were 0.27 for the low-negative standard and 1437 for the high-positive standard. Table 1 shows the predicted log MAT titers and the 95% prediction intervals for the KELA. The equivalent predicted log MAT titers for the single-read ELISA are

Table 2. Results of analysis of single-read ELISA expected log MAT equivalent titers for the 6 standards.

Sample	Expected OD	Expected log MAT titer	95% prediction interval
Low negative	0.190	−1.31	−1.78, −0.86
Negative	0.346	−0.70	−1.31, −0.11
Low positive	0.736	0.82	−0.34, 1.96
Medium positive	1.488	3.74	1.70, 5.80
Medium-high positive	2.117	6.19	3.67, 8.75
High positive	2.394	7.27	4.58, 10.02

shown in Table 2. Using distribution-based cutoff points to discriminate between positive and negative samples, both the KELA and single-read ELISA demonstrated high agreement with the MAT. The kappa statistic was 0.83, with a 95% confidence interval of 0.75–0.90 in both cases.

Indices of accuracy. The results of the logistic regression analysis are shown in Table 3. There was a significant association between the KELA slope and the seroconversion status of the animal as determined by the MAT. An increase of 0.05 in the slope is equivalent to an odds ratio of 4. The single-read ELISA ODs were also significantly associated with the ovine toxoplasmosis status as determined by the MAT (Table 3). An increase of 0.5 in the OD reading is equivalent to an odds ratio of 9.

The KELA ROC curve (Fig. 2) was essentially similar to that of the single-read ELISA (not shown). Initially, both curves revealed a rapid gain in sensitivity without much loss in specificity, followed by a phase where the gain in sensitivity was almost equivalent to the loss in specificity. The last phase showed that the loss in specificity was far greater than any gain in sensitivity. There was high agreement between the KELA and MAT results (the area under the KELA ROC was 0.968). The results of the single-read ELISA also demonstrated high agreement with the results of the MAT (the area under the single-read ELISA ROC was 0.965).

A KELA cutoff point was determined from the ROC curve to be 0.133 slope units (Fig. 2). At this cutoff point, the sensitivity of the KELA was 89% and the specificity was 96%. Similarly, the single-read ELISA cutoff point was determined from the ROC curve to

Table 3. Results of the logistic regression analysis for both KELA and single-read ELISA, showing the estimated parameters.

ELISA	Variable	Estimate	SE	*P*
Kinetics	intercept	−3.69	0.45	<0.0001
	slope	28.12	3.68	<0.0001
Single read	intercept	−3.8	0.45	<0.0001
	optical density	4.41	0.55	<0.0001

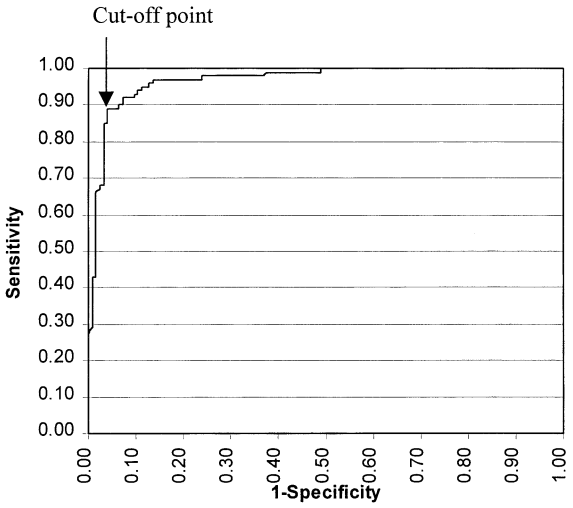

Figure 2. Receiver operating curve (ROC) for KELA to antibodies against *Toxoplasma gondii*, illustrating the optimal cutoff point.

be at the OD reading of 0.85. The corresponding sensitivity and specificity at this OD reading were 89% and 96%, respectively.

Validation of ELISAs for experimentally infected sheep. Using the KELA, group 2 ewes (inoculated with 10,000 oocysts) were seropositive by day 7 postinoculation. Group 1 ewes (inoculated with 1,000 oocysts) were seropositive by day 18 postinoculation. The criteria for seroconversion was the ROC-based cutoff point, 0.133. Similar observations with regard to seroconversion date were noted for the single-read ELISA.

Levey-Jennings charts. The KELA Levey-Jennings chart (Fig. 3) did not reveal any major systematic pattern. However, there was a slight upward trend in the slope readings for the independent standard. Almost all of the observed values were within 2 SD of the

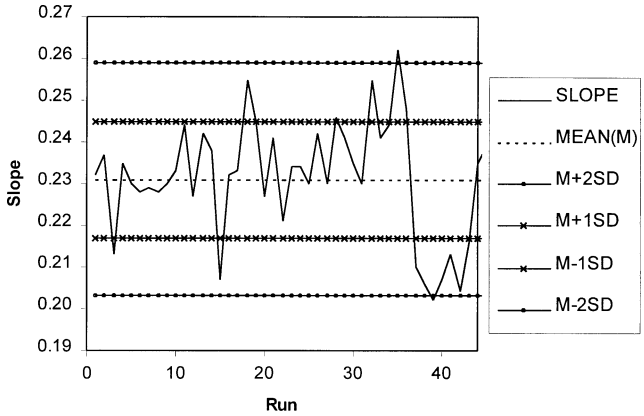

Figure 3. Levey-Jennings chart for KELA, showing the plot of the slope against the run. M+2SD = mean plus 2 (SD); M+1SD = mean plus 1 SD; M−2SD = mean minus 2 SD; M−1SD = mean minus 1 SD.

mean. Similar observations were noted for the single-read ELISA.

Discussion

A KELA and a single-read ELISA were evaluated and compared for use in the diagnosis of ovine toxoplasmosis in New York State. ELISA is a widely used tool for the diagnosis of toxoplasmosis in both animals[1,11,20,22] and humans.[13,21,24] However, before an ELISA can be used as a routine diagnostic test or as a screening test in epidemiologic studies, performance of the assay must be validated in a specific target population. Validation is necessary because different tests might have different false-positive and false-negative rates in different populations, depending on the amount of cross-reactivity.

Frequency distributions of the ELISA results from the reference sera for both the KELA and the single-read ELISA revealed 2 major groupings. One group corresponded to the presumptively infected sheep and the other corresponded to the presumptively uninfected sheep. An increase in frequency along the right slant of the peak corresponding to uninfected animals was taken as the point of intersection for infected and uninfected animals. This point was considered the cutoff point.[9] However, overlap between the animal groups constitutes a major drawback to this method of cutoff point determination.

Both the KELA and the single-read ELISA results were in close agreement with the MAT results,[3,4] as indicated by the high correlation between the KELA slopes and log MAT titers and between the single-read ELISA ODs and the log MAT titers. The agreement is further strengthened by the significant linear relationship between the ELISA results and the log MAT titers and also by a high kappa value. This finding can be interpreted as an indication that the utility of these tests as diagnostic tools for determination of exposure to *T. gondii* is the same. The only difference is the convenience of application.

Quantification of the relative amount of antibody in a sample is often requested. This amount is usually reported in the form of a titer. This quantification was attempted with both ELISAs in relation to the log MAT titers (Tables 1, 2). The predicted titers for both ELISAs were the same, which indicates that these assays are equally accurate for quantifying the amount of antibodies in a sample. This interpretation is supported by the fact that both the KELA results and the single-read ELISA results were significantly associated with the seroconversion status of the animal as determined by the MAT ($P < 0.0001$).

The ELISA ROC curves for the 2 assays were essentially the same and were close to an ideal ROC. An ideal ROC curve minimizes the phase where the gain in sensitivity is almost equivalent to the loss in specificity.[7] This phase, although brief, was observed in the curves for both ELISAs. However, both curves showed an initial rapid gain in sensitivity without much loss in specificity and a final loss in specificity that was far greater than any gain in sensitivity. The areas under both curves were very close to 1, which indicates that both assays highly agree with the MAT.

Distribution- and ROC-based cutoff points for each of the 2 assays were very close. Therefore, either method of cutoff point determination could be applied. Using ROC-based cutoff points, both the KELA and single-read ELISA registered a sensitivity and specificity of 89% and 96%, respectively. Based on these 2 parameters (specificity and sensitivity), both tests are equally valid. These findings are comparable to those reported previously, in which a sensitivity of 79% and a specificity of 100% were obtained using recombinant *T. gondii* antigens.[22]

Both the KELA and the single-read ELISA were able to detect seroconversion between 7 and 18 days postinoculation among experimentally infected ewes. This period corresponds to the appearance in sheep of anti-*T. gondii* IgG antibodies. Similar findings have been reported elsewhere.[5,26]

Repeatability (reproducibility) for the KELA was similar to that of the single-read ELISA as demonstrated by the Levey-Jennings charts. Repeatability has 2 elements: the level of agreement between 2 or 3 replicates of each sample within a run of the assay and the level of between-run agreement for the normalized values of the independent standard. Both aspects of repeatability were within reasonable limits, as has been suggested.[9]

Data on cross-reactivity between *T. gondii* and closely related apicomplexan parasites such as *Neospora, Hammondia,* and *Sarcocystis* were not generated in this study. However, negligible cross-reactions between *T. gondii* antigen and *Neospora* antisera have been demonstrated using immunoblot analysis.[17] Previous investigators showed lack of cross-reactivity between *Sarcocystis* sp. and *T. gondii*.[23] Investigation of the potential cross-reactivity between *Hammondia hammondi* and *T. gondii* demonstrated minimal cross-reaction.[14]

The performance of the single-read ELISA was as good as that of the KELA throughout the evaluation process, which consisted of examination of frequency distributions, linear regression analysis, logistic regression analysis, ROC curve analysis, sensitivity and specificity determination, use of samples from experimentally infected sheep, and use of Levey-Jennings charts for repeatability. Both assays were validated for diagnosis of ovine toxoplasmosis in New York State. These findings suggest that the 2 tests are perfect re-

placements for each other. The utility of a specific test depends on the convenience of a particular laboratory.

Sources and manufacturers

a. Microbix Biosystems, Toronto, ON, Canada.
b. Pierce, Rockford, IL.
c. Zymed Laboratories, South San Francisco, CA.
d. Parasite Biology and Epidemiology Laboratory, Beltsville, MD.
e. Nunc Maxisorb microtiter plates, Krackley, Albany, NY.
f. Ponsonby Sheep Research Station, Guelph, ON, Canada.

References

1. Buxton D: 1998, Protozoan infections (*Toxoplasma gondii, Neospora caninum* and *Sarcocystis* spp.) in sheep and goats: recent advances. Vet Res 29:289–310.
2. Dubey JP: 1990, Status of toxoplasmosis in sheep and goats in the United States. J Am Vet Med Assoc 196:259–262.
3. Dubey JP, Desmonts G: 1987, Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet J 19:337–339.
4. Dubey JP, Emond JP, Desmonts G, Anderson WR: 1987, Serodiagnosis of postnatally and prenatally induced toxoplasmosis in sheep. Am J Vet Res 48:1239–1243.
5. Esteban-Redondo I, Innes EA: 1998, Detection of *Toxoplasma gondii* in tissues of sheep orally challenged with different doses of oocysts. Int J Parasitol 28:1459–1466.
6. Harkins D, Clements DN, Maley S, et al.: 1998, Western blot analysis of the IgG responses of ruminants infected with *Neospora caninum* and with *Toxoplasma gondii.* J Comp Pathol 119:45–55.
7. Hulley BS, Cummings RS: 1988, Designing clinical research. Williams & Wilkins, Baltimore, MD.
8. Hunter D, Chadwick P, Balfour AH, Bridges JB: 1982, Examination of ovine foetal fluid for antibodies to *Toxoplasma gondii* by the dye test and an indirect immunofluorescence test specific for IgM. Br Vet J 138:29–34.
9. Jacobson RH: 1998, Validation of serological assays for diagnosis of infectious diseases. Rev Sci Tech Off Int Epizoot 17: 469–486.
10. Jacobson RH, Downing DR, Lynch TJ: 1982, Computer-assisted enzyme immunoassays and simplified immunofluorescence assays: applications for the diagnostic laboratory and the veterinarian's office. J Am Vet Med Assoc 181:1166–1168.
11. Jensen L, Petersen E, Henriksen SA, et al.: 1998, Monoclonal antibodies to *Toxoplasma gondii* strain 119 identify recently isolated Danish strains as one group. Int J Parasitol 28:1305–1313.
12. Malik MA, Dreesen DW, Cruz A: 1990, Toxoplasmosis in sheep in northeastern United States. J Am Vet Med Assoc 196:263–265.
13. Mondesire RR, Charlton DE, Tizard IR: 1981, A standardized enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Toxoplasma gondii.* J Immunoassay 2:45–57.
14. Munday BL, Dubey JP: 1986, Serological cross-reactivity between *Hammondia hammondi* and *Toxoplasma gondii* in experimentally inoculated sheep. Aust Vet J 63:344–345.
15. Owen D, Chessum BS: 1977, Observations of dye test (DT) titres to *Toxoplasma* in a non-breeding flock of sheep. Vet Rec 101:402–404.
16. Owen MR, Clarkson MJ, Trees AJ: 1998, Diagnosis of *Toxoplasma* abortion in ewes by polymerase chain reaction. Vet Rec 142:445–458.
17. Pare J, Hietala SK, Thurmond MC: 1995, An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J Vet Diagn Invest 7:352–359.
18. Payne RA, Joynson DH, Wilsmore AJ: 1988, Enzyme-linked immunosorbent assays for the measurement of specific antibodies in experimentally induced ovine toxoplasmosis. Epidemiol Infect. 100:205–212.
19. Seefeldt SL, Kirkbride CA, Dubey JP: 1989, Comparison of enzyme-linked immunosorbent assay, indirect fluorescent antibody test, and direct agglutination test for detecting *Toxoplasma gondii* antibodies in naturally aborted ovine fetuses. J Vet Diagn Invest 1:124–127.
20. Skjerve E, Waldeland H, Nesbakken T, Kapperud G: 1998, Risk factors for the presence of antibodies to *Toxoplasma gondii* in Norwegian slaughter lambs. Prev Vet Med 35:219–227.
21. Tenter AM, Johnson AM: 1991, Recognition of recombinant *Toxoplasma gondii* antigens by human sera in an ELISA. Parasitol Res 77:197–203.
22. Tenter AM, Vietmeyer C, Johnson AM: 1992, Development of ELISAs based on recombinant antigens for the detection of *Toxoplasma gondii*-specific antibodies in sheep and cats. Vet Parasitol 43:189–201.
23. Tenter AM, Vietmeyer C, Thummel P, Rommel M: 1991, Detection of species-specific and cross-reactive epitopes in *Sarcocystis* cystozoites by monoclonal antibodies. Parasitol Res 77: 212–216.
24. Tomasi JP, Schlit AF, Stadtsbaeder S: 1986, Rapid double-sandwich enzyme-linked immunosorbent assay for detection of human immunoglobulin M anti-*Toxoplasma gondii* antibodies. J Clin Microbiol 24:849–850.
25. Trees AJ, Crozier SJ, Buxton D, Blewett DA: 1989, Serodiagnosis of ovine toxoplasmosis: an assessment of the latex agglutination test and the value of IgM specific titres after experimental oocyst-induced infections. Res Vet Sci 46:67–72.
26. Wastling JM, Harkins D, Maley S, et al.: 1995, Kinetics of the local and systemic antibody response to primary and secondary infection with S48 *Toxoplasma gondii* in sheep. J Comp Pathol 112:53–62.
27. Wheeler R, Wilmore H, Savva D, Turner CB: 1990, Diagnosis of ovine toxoplasmosis using PCR. Vet Rec 126:249.